Male-female interaction and temperature variation affect pollen performance in *Citrus*

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ABSTRACT

Despite the extensive research work characterizing pollen performance in several plant species, less effort has been made to characterize it in some economically important species as *Citrus*, in which the failure of the sexual reproductive process, and subsequent parthenocarpic fruit development and seedlessness are prized characters. In this work we characterized pollen-pistil interaction in the three ancestral true-type *Citrus* species in order to determine the influence of the male and female genotypes, as well as of the temperature regime on pollen performance. Specifically, we characterized the effect of temperature on pollen grain germination *in vitro*, and on pollen tube growth *in vivo* in cut flowers and *in planta* under field temperature conditions. Results obtained showed that temperature variation has a strong effect on pollen germination, and on pollen tube kinetics and on their population size depending on the specific male-female combination. The optimum temperature for pollen germination *in vitro* was of 25 °C, while the most favorable temperature to accelerate *in vivo* pollen tube growth depended on the particular male-female interaction and ranged between 15 and 25 ºC. Furthermore, temperature appears to have an effect on self-incompatibility reaction by affecting the place where pollen tubes are arrested. Overall, our results show that pollen performance is not only an inherent characteristic of the pollen genotype, but is largely dependent on the particular male-female combination and on genotype-temperature interactions.

Keywords: Citron, Mandarin, Pollen tube growth rate, Pummelo, Self-incompatibility, Temperature stress

1. Introduction

Pollen viability and performance play an important role in the sexual reproductive success of plants, finally materialized by fertilization of the ovules and the resultant formation of seeds. Extensive research work characterizing pollen performance in different plant species revealed that pollen tube growth and its likelihood to fertilize ovule depend not only on its own genotype (Snow and Spira, 1991) but also on the genotype of the pistil (Stephenson and Bertin, 1983; Willson and Burley, 1983; Herrero and Hormaza, 1996; Hormaza and Herrero, 1996, 1999; Mulcahy et al., 1996) and on the prevailing environmental conditions during flowering and pollination (Young and Stanton, 1990; Stephenson et al., 1992; Johannsson and Stephenson, 1998; Hedhly et al., 2009; Hedhly, 2011).

Characterizing pollen performance is especially relevant for some economically important genus like *Citrus*, in which failure of the sexual reproductive process resulting in parthenocarpic fruit development and seedlessness is a prized character. The importance of *Citrus* for agriculture in a world context is shown by its worldwide distribution and its large-scale production. Grown commercially in more than 100 countries around the world in tropical and sub-tropical climates (approximately 40° N and S of the equator), citrus production (including oranges, grapefruit, tangerines and mandarins, lemons and limes) has experienced continuous growth in the last decades of the twentieth century reaching a total annual production of more than 120 million tons (FAOSTAT, 2010). Despite the economic importance of *Citrus* species little research work has been done to characterize their reproductive biology. To this it could partly contribute the lack of an established boundary between species, subspecies and interspecific hybrids, and also the paucity of knowledge of their self-incompatibility reaction and parthenocarpic behaviour. Although recent work (Distefano et al., 2011) shows that parthenocarpy in mandarin is related to an uncoupling of the onset of fruiting with the reproductive process.

On the one hand, the taxonomy of the genus *Citrus* is not exactly established. Recently, studies suggested that only three *Citrus* types, namely citron (*Citrus medica* L.), mandarin (*Citrus*

reticulata Blanco), and pummelo (*Citrus maxima* [Burm.] Merril) constituted true or valid species, and that derived from inter- and intraspecific crosses between these three species are the other important *Citrus* species such as orange, lemon, lime and grapefruit (Scora 1975, 1988; Barrett and Rhodes, 1976). The concept of the three true valid species while the other species were derived from hybridization between them is further supported from various recent studies using biochemical and molecular markers (Herrero et al., 1996; Fang and Roose, 1997; Federici et al., 1998; Nicolosi et al., 2000; Moore, 2001; Barkley et al., 2006; Deng et al., 2007).

On the other hand, little is known about the self-incompatibility and parthenocarpy behaviors of *Citrus* species, which are very important traits for fruit production and fruit quality since they result in seedless fruit. In particular, seedlessness is currently a very important feature in the evaluation of commercial citrus fruits for fresh consumption.

Also *Citrus* breeding strategies have been indeed hampered by factors associated with reproductive biology (sterility, incompatibility, nucellar embryony, juvenility). The control of self-incompatibility is thought to be gametophytic, based only on the observation of the pollen tubes growth, which usually arrest after they have covered some distance through the gynoecium (Soost, 1965). While crosses between mandarin cultivars show a clear arrest of pollen tubes in the style (Distefano et al., 2009), the incompatibility reaction has also been reported to occur along different phases of pollen tube growth through the gynoecium in different *Citrus* species (Soost, 1965; Nettancourt, 1977, 1997; Sedgley and Griffin, 1989), but more research is needed to fully characterize it as is the case for other plant families with a gametophytic self-incompatibility system such as Solanaceae (de Nettancourt 1977), Rosaceae (Sassa et al., 1993; Tao et al.,1997), Scrophulariaceae (Xue et al., 1996), Papaveraceae (Franklin-Tong et al., 1993, 1995).

In the present study, pollen-pistil interaction has been characterized in the three *Citrus* ancestral species under different temperature treatments. We first carried out a pollen germination *in vitro* experiment under five temperature regimes (10, 15, 20, 25, 30 °C), reflecting the spectrum (from cool to hot) of temperature conditions that can be found during citrus blooming season.

Second, to characterize the influence of temperature in the putative site where the pollen tube growth is arrested in self-incompatible crosses we characterized pollen tube behaviour in self-pollinated clementine. Third, to check for inter-species compatibilities and characterize the influence of the pistilar genotype and of temperature regimes on pollen tube growth, we carried out a diallel experiment (self- and cross-pollination) among the three ancestral species under four temperature regimes.

2. Materials and methods

2.1. Plant materials

In the present work four *Citrus* genotypes were used. Three genotypes belong to the three *Citrus* ancestral type species: the semi self-compatible 'Dancy' mandarin, 'Vozza Vozza' citron regarded as self-compatible, and the self-incompatible 'Sha Tian Yu' pummelo. The semi self-compatibility in 'Dancy' mandarin is not a barrier to perform this pollination experiment, since a half of pollen tubes grow normally in any pollination. The fourth and self-incompatible cultivar 'Comune' clementine (*C. reticulata* Blanco) was used as a known reference for self-incompatibility test (Distefano et al., 2009). Kim et al. (2011) reported the S genotype of several *Citrus* genotypes; however information about the genotypes used in this study is not evident. Trees, older than 10 years, of these four *Citrus* varieties were grown at the "Primosole" Experimental Farm of Catania University adopting standard cultural practices.

2.2. In vitro pollen germination

To obtain fresh pollen, 40 flowers were collected one day before anthesis from three trees of each genotype (clementine, mandarin, citron, pummelo). The anthers were removed and left to dehisce for 24 h at room temperature at about 25 °C. Fresh pollen was immediately used for testing *in vitro* pollen germination at four temperature regimes (10, 15, 20, 25, 30 °C) in Petri dishes on a solidified germination medium consisting of 100 g l^{-1} sucrose, 0.1 g l^{-1} H_3BO_3, 0.3 g l^{-1} $Ca(NO_3)_2$,

0.1 g l^{-1} KNO_3, and 10 g l^{-1} agarose (Mesejo et al., 2005). Pollen germination was arrested after 24 h by freezing at -20 °C. This procedure was adopted as it revealed its efficacy in preserving pollen morphology for microscopic observation (Hedhly et al., 2005a). Pollen was scored as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. For each treatment, germination was recorded in two Petri dishes by counting three complete fields, until reaching at least 100 pollen grains in each field, in each Petri dish (6 replicates).

2.3. In vivo pollen germination and pollen tube growth

To evaluate the effect of the genotype and of temperature regime on pollen behavior *in vivo*, a diallele experiment between the three ancestral species under field conditions and four temperature regimes was carried out. In the field experiment, flowers of clementine, mandarin, citron and pummelo were emasculated at balloon stage, hand-pollinated (10 flowers per treatment), bagged in cotton tissue, and sampled after 10 days. For the controlled chambers experiment, 120 flowers at balloon stage were collected randomly from three trees for each genotype, emasculated, and immediately placed in trays with soaked florist foam. Ten flowers per treatment were either self-pollinated or cross-pollinated in all possible combinations, except for clementine that was only self-pollinated. After pollination, trays were immediately placed in the controlled temperature chambers at 15 ºC, 20 ºC, 25 ºC and 30 °C. To compare pollen performance across experiments, all *in vitro*, *in vivo*, and *in planta* pollinations were performed with the same batch of pollen. Self- and cross-pollinated flowers of the four species were collected three days after pollination, fixed in a FAA (formalin, glacial acetic acid, 70% ethanol, 1: 1: 18 v/v; Johansen, 1940), and maintained at 4 °C until microscopic observation.

2.4. Microscopic observations

Pollen grain germination and pollen tube growth were monitored on squash preparations. Pistils fixed in FAA were washed three times in water, one hour each, and left in 5% sodium sulfite

solution overnight (Jefferies and Belcher, 1974). Pistils were, then, softened in 5% sodium sulfite solution in a microwave for 45 seconds. Before squashing the preparations, the ovary was cut from the stigma-style, and both parts were further cut longitudinally and split into two parts. Following the staining procedure and squashing with 0.1% aniline blue in 0.1 N K_3PO_4 (Linskens and Esser, 1957), the preparations were observed under a fluorescence microscope (Leica DM 2500 of Leica Microsystems GmbH using I3 filter excitation 450-490 nm, Wetzlar, Germany). Pollen tube growth was recorded as the length of the longest pollen tube in the stigma-style, measured in 10 pistils/genotype. The number of pollen tubes reaching the base of the style was also recorded.

2.5. Statistical analyses

For statistical analysis, to normalize data and homogenize variances, all count data were square-root-transformed before carrying out the analyses of variance. In the case of pollen germination *in vitro*, the removal of 10 ºC observations allowed a better normalization of data. In the case of pollen tube numbers at the base of style under growth chamber conditions, the analysis was carried out only among the crosses in which some pollen tubes were reported. Statistical analyses were done using R 2.13.1 (The R Foundation for Statistical Computing, 2011; http://www.R-project.org).

3. Results

3.1. Pollen germination in vitro

The effect of incubation temperature on the *in vitro* pollen germination of the four *Citrus* varieties was expressed as percentage of pollen grains germinated during 24 hours (Fig. 1). Apart from significant main effects, the analysis of variance revealed a highly significant genotype-temperature interaction (Table 1). No pollen germination was observed for any of the *Citrus* genotypes at the lowest temperature of 10 °C. Pollen grain germination was less than 10% at 15 °C except for clementine in which still no germination was observed. Bigger and more significant

differences were observed at 20 °C when pollen grain germination ranged from 3% for clementine to 22% for citron and pummelo. Mandarin, citron and clementine showed a maximum germination percentage at 25 °C ranging from 28 to 45%. Also pummelo pollen grains showed the highest germination percentage (>90%) at 25 °C. Examining the global trend of pollen germination in response to increasing temperature, a progressive increase in pollen grain germination rate from 15 °C to 25 °C can be observed for all genotypes, followed by a sudden decrease when temperature reached 30 °C except for clementine.

3.2. Interspecific crosses in field conditions

Ten days after self and cross-pollination under field temperature conditions, pollen tubes reached the base of the style in most combinations except for self-pollinated pummelo (black bars in Fig. 2). The length of distance travelled by pollen tubes from the stigma to the ovary varied between species from 5.7 mm (0.9 mm SD) in mandarin, 5.9 mm (1.1 mm SD) in clementine, 12.1 mm (1.3 mm SD) in pummelo, to 14.1 mm (1.7 mm SD) in citron. Although pollen tubes in the self-pollinated citron only reached 60% of the length of the style, a few pollen tubes were observed at the base of the style in a few flowers. In all other combinations pollen tubes reached the base of the style in all flowers and varying number of tubes were recorded ranging from 2 to 40. Thus a highly significant difference between the crosses carried out was found for pollen tube growth and for the number of pollen tubes reaching the ovary (Table 1). Interestingly, the number of pollen tubes at the base of style was not the same in reciprocal crosses, and varied depending on the particular male-female or female-male combination. As a female receptor, pummelo and citron had significantly more pollen tubes at the base of their styles compared to mandarin. However, as a male donor, none of the species was more high performing in more than one combination. As a particular male-female combination, pummelo x mandarin and citron x pummelo resulted in the highest number of pollen tubes (>40) reaching the base of the style.

3.3. Interspecific crosses at different temperature regimes

Subjecting flowers to varying temperatures between 15 and 30 ºC in controlled growth chambers differentially affected pollen tube growth in the style, and the number of pollen tubes reaching the base of style. The analysis of variance revealed not only a highly significant effect of the crosses made (genotype and combination) and of temperatures assayed, but also of their interaction (Table 1). Thus, pollen performance depended on the particular male-female or female-male combination. Overall, 20 ºC, and to a lesser degree 25 ºC, promoted pollen tube elongation; 15 ºC and to a lesser extent 30 °C were inhibitory in most cases. The promoting effect of 20ºC can best be seen by the number of pollen tubes reaching the base of style of mandarin, and in the combination citron x pummelo 3 days after pollination (Fig. 3).

Overall the longest pollen tubes were observed in citron styles and, at a less degree, in cross-pollinated pummelo styles. Nevertheless, due to the different stigma + style length of the considered species, pollen tubes covered the whole style length in mandarin after three days. Pollen tube growth rate was also very effective in citron x pummelo combination in which, despite the very long style length, a number of pollen tubes reached the base of the style after three days at different temperatures.

Furthermore, temperature appears to have a significant effect on the place where pollen tubes were arrested in self-incompatible pollinations (clementine and pummelo). For clementine, while at 15 and 25 ºC pollen tubes were arrested before reaching one third of the style length, they traveled significantly longer distance at 20 ºC (about two third of the length of style), and less distance -although not significantly different from 15 ºC and 25 ºC- at 30 ºC (25% of the length of style).

Comparing mandarin and citron as female recipients, the effect of temperature appeared to be independent of the pollen donors in the first, but varied with the pollen donor in the second. As a pollen donor, no single species was consistently more high-performing in more than one combination. Under our controlled temperature conditions (maintaining flowers for 3 days after

pollination) only the three combinations in which mandarin was used as a female recipient, and the combination citron x pummelo showed some pollen tubes at the base of the style.

4. Discussion

4.1. Male-female interaction and pollen performance

To analyze the influence of the genotype on pollen performance we carried out first an *in vitro* pollen germination assay to assess pollen viability under different temperature regimes. All the analyzed *Citrus* species showed more or less the same pollen grain germination trend as temperature increased from 10 ºC to 30 ºC, but different pollen grain germination rate at the same temperature. Furthermore, the ranking of the different genotypes studied was not constant and varied with temperature, which resulted in highly significant genotype-temperature interaction. Overall, the pollen had a sufficient viability, and at optimum temperature pollen germination ranged from >90% for pummelo to 28% for clementine. Variation in *in vitro* pollen germination between different genotypes within the same species and at the interspecific level has been reported in other species reflecting either differences in the genetic background or the origin and adaptation of the pollen-producing parent (reviewed in Hedhly, 2011).

Pollen performance *in vivo* (expressed as pollen tube length in the style and number of pollen tubes reaching the base of style) varied, as well, depending on the genotype of the pollen donor and of the female receptor.

Under field temperature conditions, the ten days after pollination allowed pollen tubes to reach the base of style of all flowers and in all combinations except for self-pollinated flowers of pummelo and citron. Thus, results obtained herein showed that, under field temperature conditions, and based on the number of pollen tubes at the base of style, there is no particular pollen donor or female recipient that is consistently high-performing, but the outcome is rather dependent on the particular male-female and/or female-male combination. Under both field temperature and growth chamber conditions, those combinations that promoted more pollen tube growth in the style and

higher number of pollen tubes at the base of style, can be considered compatible pollinations. Thus, based on these results, 'Sha Tian Yu' pummelo can be considered self-incompatible, while the small number of pollen tubes reaching the base of style of self-pollinated 'Vozza Vozza' citron point out either to be a self-compatible slow progamic phase, or to be a late acting self-incompatibility; however, more experiments are needed to have a definitive answer.

Examining the differential behaviour in pollen performance, although some female recipients were somehow deciding factor in more than one combination irrespective of the male genotype, overall, no single pollen donor was consistently more high performing, and pollen performance was definitively influenced by the particular male-female or female-male interaction. Differences in pollen performance depending on the pollen and the pistil genotypes have been previously reported in other species (e.g. *Prunus* spp.: Hedhly et al., 2005a; *Citrus* spp.: Distefano et al., 2009). Although in some species the male genotype was the deciding factor in pollen performance and the ranking was consistent across different female genotypes (Charlesworth et al., 1987; Snow and Spira, 1991; Marshall, 1998; Mitchell and Marshall, 1998), results obtained in this and other works (Stephenson and Bertin, 1983; Vaser et al., 1987; Cruzan, 1990; Willson, 1994; Herrero and Hormaza, 1996; Kerwin and Smith-Huerta, 2000; Marshall and Diggle, 2001; Hedhly et al., 2005a) suggest that the outcome of the sexual phase is rather dependent on the specific male-female genotype interaction. Also the different rate of compatibility for pollen tube behavior in interspecific cross condition, are consistent with those obtained in previous works on citrus (Ton and Krezdorn, 1966; Ngo, 2001; Yamamoto, 2006; Distefano et al., 2009).

4.2. Temperature variation effects on pollen performance

One of the most important environmental factors that could affect pollen performance is the temperature regime during the progamic phase spanning from pollination to fertilization. It has been shown that temperature affects pollen germination (Elgersma et al., 1989; Shivanna et al., 1991), and pollen tube kinetics in the style (Lewis, 1942; Jefferies et al., 1982; Elgersma et al., 1989;

Hedhly et al., 2005b). Studies in sweet cherry (*Prunus avium* L.) showed that temperature affects not only pollen tube growth rate, but also pollen tube dynamics and, as a consequence, influences the amount of the pollen tubes that succeed in reaching the ovary (Hedhly et al., 2005a).

Our results of pollen germination under different *in vitro* incubation temperatures, showed a progressive increase in germination rate from 15 °C to 25 °C, with a sharp decrease when temperature reached 30 °C. All three true ancestral species plus clementine showed their maximum germination percentage at 25 °C. This is in line with results reported for different sweet orange cultivars (Ramos et al., 2008). Thus, the absence of germination at 10 ºC, and the optimum germination temperature of 25 ºC reflect the subtropical origins of all *Citrus* species examined. Similar relatively high optimum temperatures were reported for other subtropical and tropical species such as *Pistacia* spp. (Acar and Kakani, 2010), olive (Koubouris et al., 2009), and mango (Sukhvibul et al., 2000), while relatively lower optimum temperatures in the range of 15-20 °C are usually reported for temperate fruit trees like apricot (Egea et al., 1992), sweet cherry (Pirlak, 2002), and sour (Cerovic and Ruzic, 1992).

Our results confirmed the self-incompatibility of clementine (Ton and Krezdorn, 1966; Distefano et al., 2009) and pummelo (Ngo, 2001; Yamamoto, 2006) showing pollen tube growth arrest in the first quarter of the style following self-pollination under field temperature conditions. However, characterizing pollen tube growth in these self-incompatible crosses and under different temperature regimes, revealed significant differences in the place where pollen tubes were arrested. An effect of temperature on the self-incompatibility reaction was reported in pear (Lombard et al. 1972). In *Citrus* species, the incompatibility reaction was classified by the degree of self-incompatibility at different levels of the style (Ngo, 2001; Yamamoto, 2006). In some cases, pollen tubes are arrested very soon at the stigma level (Ton and Krezdorn, 1966), and, in others, they are arrested at the base of the style, in the ovary or ovules (Sage et al., 1994; Gòmez et al., 2004). The influence of temperature reported here on the place where pollen tube are arrested, can explain part

of the conflicting reports in the literature, and suggest a superimposed influence of temperature and also of the specific male and female genotypes interaction.

In compatible pollinations, pollen performance in the style (pollen tube growth and the number of pollen tubes reaching the base of style) did not correspond to pollen performance *in vitro*. This discrepancy can be explained by an effect of a suboptimal artificial germination medium, by a different optimum temperature between pollen germination and pollen tube growth as discussed below, or by an influence of the female recipient used (Hedhly et al., 2005a). Results obtained herein show an optimum temperature for pollen germination *in vitro* of 25 °C, while the most favourable temperature to accelerate *in vivo* pollen tube growth depended on the particular male-female interaction and ranged between 15 and 25 ºC. This result is in line with previous reports of a different optima for both parameters (McKee and Richards, 1998; Kakani et al., 2002), suggesting that they are independent processes.

Overall, our result showed that increasing temperature increased pollen germination and pollen tube growth, and that both low and high temperature had a negative effect. Results obtained in this work show how pollen performance is not only an inherent characteristic of the pollen genotype, but is largely dependent on male-female and genotype-temperature interactions.

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Figures and table legends

Fig. 1. *In vitro* pollen grain germination percentage at different incubation temperatures (10 ºC, 15 °C, 20 °C, 25 °C, and 30 °C for 24h) for ‘Dancy’ mandarin, ‘Vozza Vozza’ citron, ‘Sha Tian Yu’ pummelo and ‘Comune’ clementine. (means ± se).

Fig. 2. Pollen tube growth in the styles of cross- and self-pollinated flowers of ‘Dancy’ mandarin (M), ‘Vozza Vozza’ citron (C) and ‘Sha Tian Yu’ pummelo (P), 10 days after pollination under field temperature conditions. Upper: pollen tube length (black bars) in the stigma-style (grey bars); lower: number of pollen tubes at the base of the style. (means ± se).

Fig. 3. Pollen tube growth in the styles of cross- and self-pollinated flowers of ‘Dancy’ mandarin (M), ‘Vozza Vozza’ citron (C), ‘Sha Tian Yu’ pummelo (P), and ‘Comune’ clementine (Cl) 3 days after pollination under incubation temperatures of 15 °C, 20 °C, 25 °C, 30 °C. Upper: pollen tube length in the stigma-style. Horizontal black lines indicate the stigma-style length (5.7 mm for ‘Dancy’, 12.1 mm for ‘Sha Tian Yu’, 14.1 mm for ‘Vozza Vozza’ citron and 5.9 mm for 'Comune' Clementine); lower: number of pollen tubes at the base of the style. (means ± se).

Table 1

Analyses of variance of temperature effect on pollen germination *in vitro*, pollen tube growth in the style and number of pollen tubes at the base of the style under field conditions and growth chamber constant temperatures.

Table 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Pollen germination in vitro						
[a]Temperature	3	297.5	99.2	601.8	< 2.2e-16	***
Genotype	3	98.6	32.9	199.4	<2.2e-16	***
Temperature:genotype	9	71.5	7.9	48.2	<2.2e-16	***
Residuals	80	13.2	0.2			
Pollen tube growth in the style in the field						
Cross	8	35.8	4.5	121.3	<2.2e-16	***
Residuals	81	3.0	0.04			
Number of pollen tube at the base of the style in the field						
Cross	8	381.5	47.7	275.92	<2.2e-16	***
Residuals	81	14.0	0.2			
Pollen tube growth in the style in growth chambers						
Cross	9	115.9	12.9	490.3	<2.2e-16	***
Temperature	3	35.2	11.7	447.3	<2.2e-16	***
Cross:temperature	27	19.2	0.7	27.1	<2.2e-16	***
Residuals	359	9.4	0.03			
Number of pollen tube at the base of the style in growth chambers						
[b]Cross	5	156.6	31.3	29.7	3.1e-14	***
Residuals	53	55.9	1.0			

[a] *The 10ºC temperature observations was removed from the analysis*
[b] *Anova was done only on those 6 crosses with pollen tubes at the base of the style*

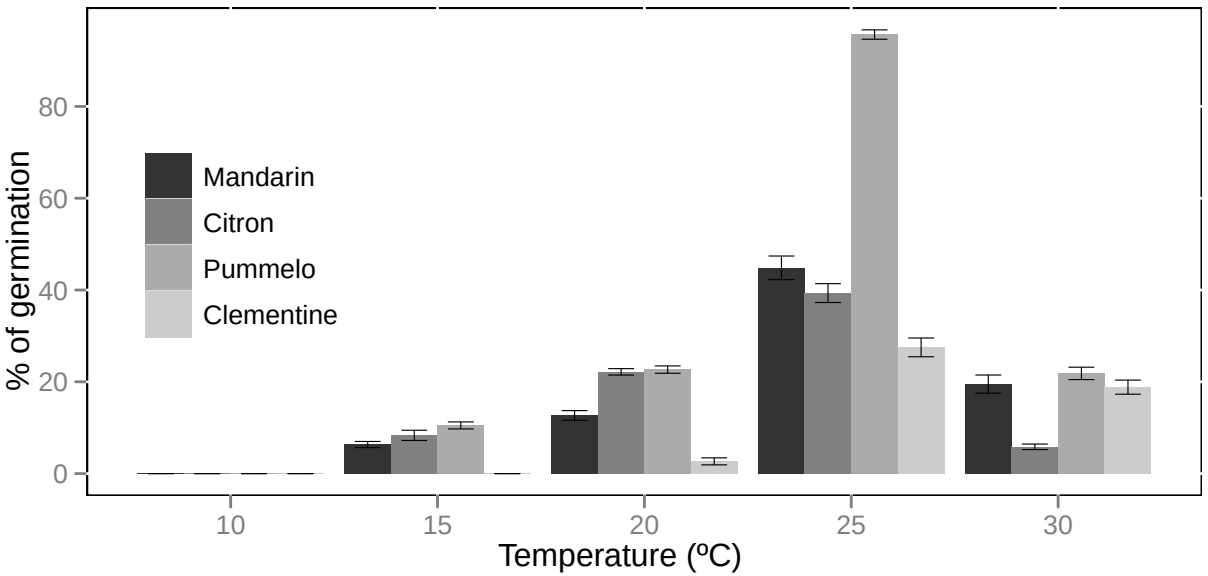

% of germination
0
20
40
60
80
10
15
20
25
30
Temperature (ºC)
Mandarin
Citron
Pummelo
Clementine

Fig. 2

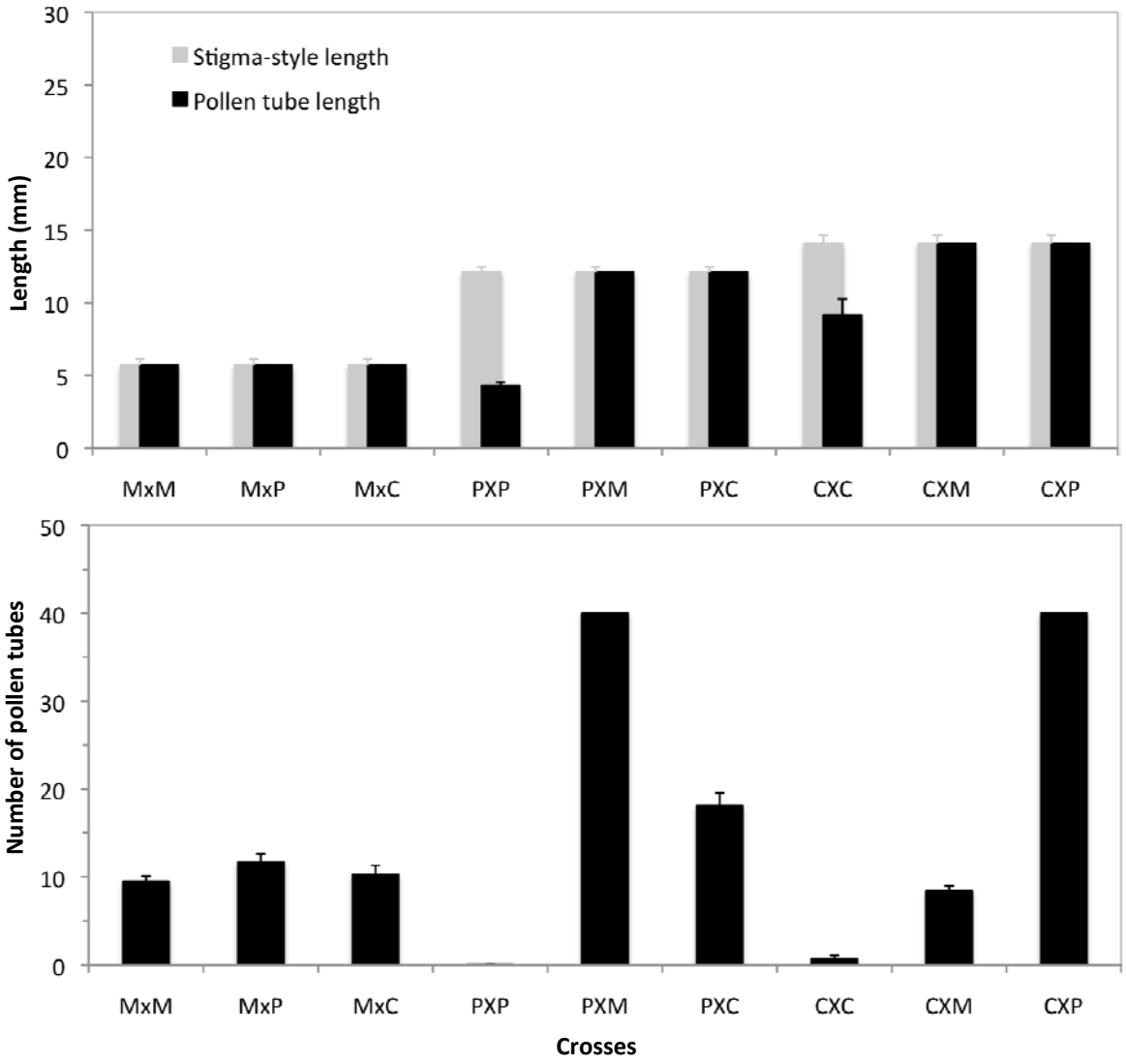
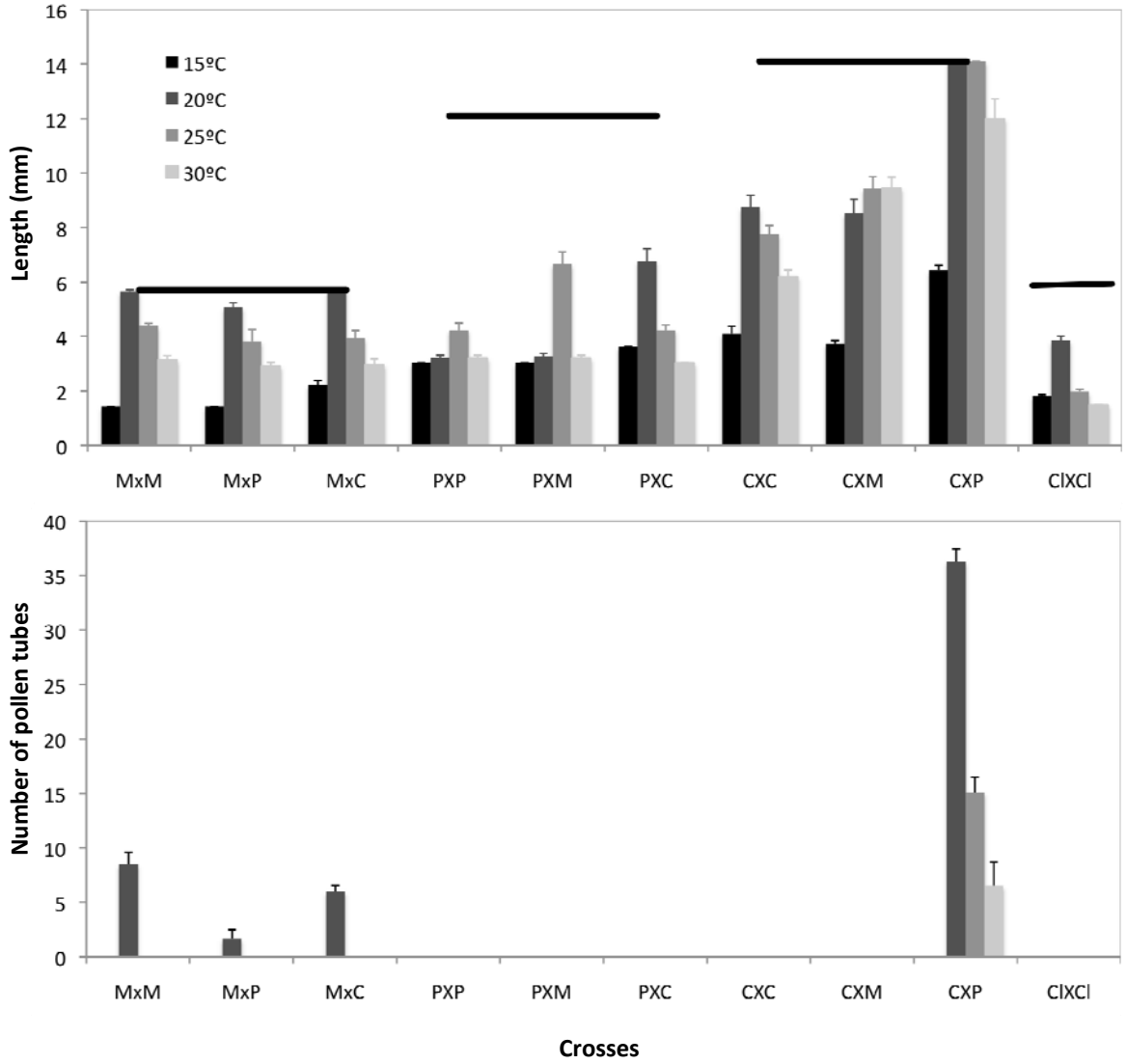

Stigma-style length
Pollen tube length
Length (mm)
30
25
20
15
10
5
0
MxM
MxP
MxC
PXP
PXM
PXC
CXC
CXM
CXP
Number of pollen tubes
50
40
30
20
10
0
MxM
MxP
MxC
PXP
PXM
PXC
CXC
CXM
CXP
Crosses

Fig. 3


Length (mm)
15ºC
20ºC
25ºC
30ºC
MxM
MxP
MxC
PXP
PXM
PXC
CXC
CXM
CXP
ClXCl
Number of pollen tubes
Crosses